THE IMMUNOLOGICAL RELATIONSHIP OF HORDEIN OF BARLEY AND GLIADIN OF WHEAT AS SHOWN BY THE COMPLEMENT FIXATION, PASSIVE ANAPHYLAXIS, AND PRECIPITIN REACTIONS.*†

THE BIOLOGICAL REACTIONS OF THE VEGETABLE PROTEINS. IV.‡

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There are numerous observations in the literature on biological reactions with extracts of vegetable substances, showing that such extracts, containing mixtures of the soluble vegetable proteins of the plant material employed, are capable of acting as antigens which will react with, and incite the formation of, antibodies demonstrable by precipitin, complement fixation, and anaphylaxis reactions. Much of this literature is reviewed in the first article of this series and it concerns especially the precipitin reaction. None of this literature refers to experiments performed with isolated proteins, with the exception of casual observations by Jacoby and by Osborne, Mendel, and Harris, that the serum of animals immunized with purified ricin gives precipitates with ricin. At that time we found no publications on the occurrence of complement fixation reactions with vegetable antigens, except observations by Dunbar on pollens. Since then this subject has received some consideration, the literature of which is here reviewed.

Ballner[1] reports that strong and specific complement fixation reactions can be obtained with solutions made by extracting the ground grains, etc., with physiological salt solution, heating the extract 5 hrs. at 56° C., and then filtering. He describes these extracts as giving the chemical reactions characteristic of albumoses. The serum of rabbits immunized with these solutions gave fixation reactions indicating a distinct

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‡ The preceding articles of this series are: I. Wells and Osborne, *Jour. Infect. Dis.*, 1911, 8, p. 66; II. Wells and Osborne, *ibid.*, 1913, 12, p. 341; III. White and Avery, *ibid.*, 1913, 13, p. 103.

[1] *Sitzungsber. kaiserl. Akad., Wien*, 1910, 119, Abt. 3, p. 17.

"group specificity." Thus, anti-wheat serum gave reactions with wheat extract in dilutions of 1–20,000, and with rye extract 1–10,000; reactions were also given with extracts of barley and oats in considerably greater concentration, but extracts of rice and corn reacted with wheat antiserum only in extreme concentrations, as did also the extracts of peas and lentils. Pea antiserum was very specific, reacting with pea extract at 1 to 40,000 dilution, and with lentil extract only in dilution of 1 to 50. Since these extracts used by Ballner contained several of the proteins present in the materials extracted, it is impossible to determine which of these caused the reactions.

Dunbar,[1] in his work on "hay fever," found that various pollens can be differentiated from one another and from other parts of the same plant by the complement fixation reaction.

Wendelstadt and Fellner[2] found that extracts of leaves of various fruit trees, as well as extracts of leaves of cereals, gave unsatisfactory results by the complement fixation reaction, but that simple saline extracts of the seeds of beans and peas gave positive reactions of certain well defined degrees of specificity. Antiserum for extracts of the beans of *Vicia faba* reacted with similar extracts from *Vicia sativa* and *Pisum sativum*, but not with those of *Phaseolus vulgaris* or *Phaseolus multiflorus;* antiserum for seeds of *Pisum sativum* reacted with extracts of *Vicia sativa* and *Vicia faba*, but not with those of *Phaseolus;* antiserum for *Phaseolus grandiflorus* reacted with extracts of *Phaseolus vulgaris*, but not with extracts of *Pisum sativum*, *Vicia sativa*, or *Vicia faba*. However, the reactions with the heterologous extracts were always less strong than with the homologous extracts, except in the case of *Phaseolus*. These results may be compared with those which they obtained by the precipitin reaction. A very strong precipitating antiserum for extracts of the seeds of *Vicia faba* gave almost as strong precipitating reactions with extracts of *Pisum sativum* and *Vicia sativa* as with extracts of *Vicia faba*, but none with extracts of *Phaseolus vulgaris* or *grandiflorus*, thus agreeing with the complement fixation reaction. Furthermore, experiments by means of passive anaphylaxis showed that rabbit antiserum for extracts of *Pisum sativum* made guinea-pigs sensitive to *Vicia faba*, but not to either variety of *Phaseolus*. Peculiar results were obtained with the serum of rabbits immunized with extracts of green leaves; thus, antiserum for pear leaves reacted more strongly with extracts of plum and peach leaves than with extracts of pear leaves themselves; antiserum for barley leaves reacted strongly with the extracts of leaves from wheat and rye, but only slightly with extracts of barley leaves. Feeding rabbits for some time with peas, corn, or potatoes did not cause the appearance in their blood of appreciable amounts of antibodies causing either precipitin or complement fixation reactions.

According to Sauli[3] the conglutination reaction gives sharper results with antisera for vegetable proteins than does the precipitin reaction, and he therefore applied this reaction to a number of plant extracts. He found that antiserum for extracts of seeds of *Brassica rapa rapifera*, which gave practically no reaction with extracts of seeds of *Pisum sativum* or *Trifolium pratense*, gave about as strong a reaction with extracts of the seeds of *Brassica napus rapifera* as with the homologous extract; in general, this serum gave strong reactions with extracts of seeds from all plants of the family *Cruciferae*, but very weak or none at all with *Papilionaceae*, thus showing a strong group reaction for botanically related species. Antiserum for *Vicia faba*

[1] *Ztschr. f. Immunitätsf.*, 1910, 4, p. 740.

[2] *Ibid.*, 1910, 8, p. 43.

[3] *Ibid.*, 1911, 9, p. 359.

aquina gave an almost equally strong reaction for *Vicia faba atropurpurea* but considerably weaker for *Pisum sativum*.

An interesting observation is described by Pick,[1] to the effect that an immune serum containing precipitins for edestin has no effect upon edestin crystals suspended in it, but if sufficient salt is added to cause a little of the edestin to go into solution, immediate precipitation occurs.

Recently White and Avery,[2] working with the same preparations that have been used in other experiments described in this series of articles, obtained a precipitin for edestin which gave strong reactions with solutions of edestin diluted to 1 to 10,000, but did not react with gliadin in even twice as great a concentration; specific complement fixation was also obtained with the serum of rabbits immunized with edestin, but no reaction was obtained with the same serum and gliadin.

Chapman,[3] in an address, states briefly that experiments have been performed in his laboratory with extracts made with 10 per cent NaCl solution from seeds of 15 species of *Acacia*, the extracts being heated at 55° C. for 3-6 hours for sterilization. Extracts of 11 out of the 15 different species of seeds gave precipitates with normal serum. Extracts of *Acacia pycnantha* were used for immunizing, and the resulting antiserum gave precipitin reactions with extracts of all the acacias, but none with extracts of leguminosae (*Pisum*, *Vicia*, *Phaseolus*) nor of wheat, oats, or other unrelated forms.

Lusini[4] reports the production of specific precipitins for *Lytta vesicatoria*, *Smilax officinalis*, *Althaea officinalis*, *Jateorrhyza palmata*, and *Digitalis purpurea;* uncertain results were obtained with *Aloes*, and negative results with *Rheum palmatum* and *Picraene excelsa*. This work was done with the object of applying the immunity reactions to the identification of botanical drugs.

To complete the review of the literature with indirectly related observations, we mention that Rosenblatt-Lichtenstein[5] found that immunization with *Algae* of different species produces quantitatively specific agglutinins. Gallio-Valerio and Bornand[6] immunized rabbits with extracts of *Agaricus muscarina Linn.* and obtained precipitins which differentiate this from *Boletus edulis* and other mushrooms. Thornton[7] also states that plant and animal cells have opposite electrical charges, animal cells being negative and plant cells positive, but that the contact of the two types of cells does not result in a mutual discharge, from which fact he concludes that the charge is not in or on the cell membranes, but in the cytoplasm.

There is much evidence in support of the hypothesis that the precipitin, agglutinin, complement fixation, and anaphylaxis reactions all represent the interaction of one and the same specific immune body with its corresponding antigen, the different reactions being merely different methods of demonstrating the presence of

[1] Kolle and Wassermann, *Handbuch d. path. Mikroorg.*, 1912, 1, p. 689; the work is credited to Obermayer and Pick but no reference is cited.

[2] *Jour. Infect. Dis.*, 1913, 13, p. 103.

[3] *Proc. Linnean Soc.*, New South Wales, 1910, 35, p. 549.

[4] *Atti. R. Fisiocrit.* (Siena), 1912, 219, p. 147.

[5] *Arch. Anat. u. Physiol.*, 1912, Phy. Abt., p. 415.

[6] *Ztschr. f. Immunitätsf.*, 1913, 17, p. 180.

[7] *Proc. Roy. Soc.* (B), 1910, 82, p. 638.

this antibody. Observations have been made, however, which are not in harmony with this simple interpretation, so that at present this view cannot be considered as established.

EXPERIMENTS.

Since hordein from barley (*Hordeum vulgare*) and gliadin from wheat (*Triticum vulgare*) or from rye (*Secale cereale*) are similar in their physical properties, in the proportion of their products of hydrolysis, and have been shown to be closely related to one another by the anaphylaxis reaction,[1] we have undertaken to study the complement fixation, the precipitin, and the passive anaphylaxis reactions exhibited by these physically and chemically unique proteins.

In all our experiments the following methods were used:

A. *Passive anaphylaxis.*—The serum or defibrinated blood from an immunized rabbit, in doses varying from 0.5 to 3.0 c.c., was injected into the peritoneum of guinea-pigs (200–300 gm.) and the antigen was injected into these in doses of 0.1 gm. (or 1 c.c. fluid antigen) after an interval of from 24 hrs. to 4 days.

B. *Precipitin test.*—Different quantities of the protein to be tested were used with 0.1 c.c. of the unheated serum of the immunized animal. Similar series of experiments with the serum of normal animals of the same species, and corresponding amounts of antigen, were used as controls.

C. *Complement fixation.*—The antigen was used in different amounts: of the antiserum, 0.1 c.c.; of the complement (guinea-pig), 0.05 c.c.; of sheep corpuscle amboceptor, twice the unit amount; of 5 per cent washed sheep corpuscles, 1 c.c. For controls, similar series were run with normal serum; and also the usual controls of antigen and amboceptor were made. Usually the serum was inactivated, but this seemed to make no difference; in final tests inactivated serum was always used.

Some difficulty was encountered in the production of the antiserum on account of the relatively slight solubility of the proteins, and especially of the gliadin. In the earlier work the intravenous method of injecting was used, but on account of the loss of animals through embolism, anaphylactic shock, etc., this method was finally dropped and the intraperitoneal route used entirely, as this seemed to give just as potent an antiserum, without loss of animals.

ABSTRACT OF PROTOCOLS.

Experiment 1.—Gliadin, wheat. During a period of 23 days a rabbit was given six intravenous injections containing in all 1.2 gm. of gliadin dissolved in 0.1 per cent

[1] See preceding paper, II, *Jour. Infect. Dis.*, 1913, 12, p. 341.

NaOH, but it died suddenly immediately after the last injection. (In all our experiments the vegetable protein solutions were prepared just before injecting.) Blood drawn on the sixteenth and twenty-third days gave no precipitin reaction with wheat gliadin in dilutions varying from 1–400 to 1–4,000.

Experiment 2.—Gliadin, wheat. A rabbit received during the course of 7 weeks 10 intravenous injections, containing in all 2 gm. of gliadin dissolved in 0.1 per cent NaOH, the dates of the injections being as follows: February 13, 19, 23, and 26, March 1, 8, 9, and 22, and April 1 and 10. The rabbit died on April 30 in poor condition.

Precipitin tests were made with dilutions of 1–400 up to 1–400,000, but negative results were obtained with blood drawn on the following dates: March 1, 8, and 16.

Complement fixation tests were made on April 13, and positive reactions obtained with dilutions of 1–400 to 1–4,000, the only dilutions tried.

Passive anaphylaxis experiments were tried on April 16 and 23; each time 3 guinea-pigs were given an intraperitoneal injection of the rabbit's blood (from 1 to 3.5 c.c. to each animal), but none of these animals reacted to gliadin injected 48–72 hrs. later.

Experiment 3.—Gliadin, wheat. During 27 days a rabbit received 8 intraperitoneal injections containing a total of 4.7 gm. of wheat gliadin dissolved in 0.1 per cent NaOH, spaced as follows: June 19, 24, 27, July 1, 5, 8, and 15. On July 22 blood was drawn and the complement fixing power determined. Positive reactions were obtained with wheat gliadin in dilutions up to 1–10,000. Negative results were obtained with hordein and rye gliadin in dilutions of 1–2,000. As another rabbit at this time was furnishing a serum of much higher power, nothing further was done with this animal.

Experiment 4.—Gliadin, wheat. A rabbit received injections containing gliadin on the same dates and with the same amounts as in Experiment 3. Ten days after the last injection it was bled to death, and the serum used for experiments. On July 22 a sample of blood was drawn and found to give a positive complement fixation reaction with wheat gliadin in a dilution of 1–100,000, while negative reactions were given with hordein, rye gliadin, wheat "proteose," and malt "proteose" in dilutions of 1–2,000. Positive complement fixation was given with wheat gliadin on July 29, with dilutions of 1–20,000, while no reactions were obtained with the above-mentioned heterologous proteins at 1–2,000. (More concentrated solutions cannot be used safely as a routine practice, because stronger solutions of these vegetable proteins often yield precipitates with serum, but occasionally, as will be noted, we have been able to make tests with strengths as great as 1–400.) Precipitin tests were made with this serum, but no precipitin reaction was obtained with wheat gliadin in a dilution as low as 1–1,000.

A test of passive anaphylaxis was made by injecting 3 c.c. of this serum (intraperitoneally) into each of 5 guinea-pigs. Forty-eight hours later 0.1 gm. wheat gliadin was injected into each with negative results.

Experiment 5.—A 5 per cent solution of hordein, dissolved in 0.1 per cent NaOH solution, was injected into the ear vein of a rabbit; 7 injections, containing a total of 2.63 gm. of hordein, were thus given during 26 days. The animal died suddenly after the last injection. Serum obtained on the twenty-third day, after 5 injections had been made, gave no precipitin reaction with hordein in dilutions of 1–2,000 or greater.

Experiment 6.—Hordein was injected as in Experiment 5, and the rabbit was bled to death 17 days after the last injection. Precipitin tests were made on the twenty-third, thirty-fourth, and forty-fifth days, all being negative with hordein itself, in dilutions varying from 1–1,000 to 1–800,000, as well as with wheat gliadin, rye gliadin, wheat "proteose," and malt "proteose."

On the forty-second day the serum gave a positive complement fixation with hordein in dilutions up to 1–400,000, but no reaction with the above-mentioned heterologous proteins in dilutions of 1–2,000 and up. Again, on the forty-fifth day, positive results were obtained in dilutions of 1–600,000 of hordein, but with the heterologous proteins negative results were given in dilutions of 1–2,000 and up. Negative results were also obtained when an attempt was made to produce passive anaphylaxis as follows: On the thirty-fifth day blood was drawn and injected intraperitoneally into 6 guinea-pigs, the doses ranging from 0.5 to 2.5 c.c. Two to three days later 0.1 gm. hordein was injected intraperitoneally with negative results. Negative results were also given by 8 other guinea-pigs receiving 2 c.c. of serum drawn on the forty-fifth day, and, from 2 to 5 days later, an intraperitoneal injection of 0.1 gm. hordein.

Experiment 7.—Hordein was given to the amount of 3.5 gm. in 7 doses during a period of 22 days. Seven days after the last injection a sample of serum gave positive complement fixation in dilutions up to 1–200,000 with hordein, but not with wheat and rye gliadin in dilutions of 1–2,000. No other experiments were performed with this animal.

Experiment 8.—Injections were made as in Experiment 7. A sample of serum obtained on the twenty-ninth day gave a positive complement fixation reaction in dilutions up to 1–300,000 with hordein, but none with wheat or rye gliadin, wheat "proteose," or malt "proteose." On the thirty-second day the serum gave a positive fixation reaction with hordein in dilutions up to 1–500,000, but not beyond. No reactions were given with the heterologous proteins in dilution of 1–2,000. No precipitin reaction was obtained when this serum was tested with hordein in dilutions as low as 1–1,000, nor with the above-mentioned heterologous proteins. Here again no passive anaphylaxis could be obtained when 0.1 gm. hordein was injected into each of 5 guinea-pigs, 48 hrs. after they had received 3 c.c. of the serum intraperitoneally.

Experiment 9.—Hordein was injected into a rabbit intraperitoneally, as follows: 0.5 gm., February 17; 0.6 gm., February 21; 0.75 gm., February 25; 0.75 gm., February 28; 1 gm., March 2; 1 gm., March 7 ; 1 gm., March 10. Ten days later the animal was killed and the serum collected. It was found to give a complement fixation with hordein in 1–1,000,000 dilution and also to wheat and rye gliadin in the same dilution. With wheat proteose the reaction was negative, while with malt proteose it was positive in a 1–500,000 dilution. This antiserum also gave a precipitin reaction, as follows: with hordein in 1–100,000, with wheat gliadin 1–200,000, and with rye gliadin 1–40,000, but with wheat and malt proteose the reaction was negative. The passive anaphylactic reaction was also positive, a slight reaction being obtained with hordein and wheat gliadin, and a moderate reaction with rye gliadin.

It will be noted that the results obtained in Experiment 9 are decidedly different from those of the eight preceding experiments, in that the precipitin and passive anaphylactic reactions, previously not demonstrable, were now definitely positive, and in that

at the same time the apparent marked specificity shown in the earlier experiments had been so altered that the closely related protein gliadin reacts with the antihordein serum. (We are unable to explain why a stronger reaction was obtained with wheat gliadin than with hordein, and suspect some undetected error.)

Experiment 10.—This was a duplicate of the preceding experiment, the same doses being given at the same time, etc. It is of interest in that the animal's serum was of slightly lower titre, particularly as regards the precipitin test, and at the same time the passive anaphylactic reaction was positive only with hordein. In other words, this weaker serum showed more tendency to specificity, and a narrower range of reaction.

Inasmuch as gliadin and hordein differ from most other proteins in being soluble in alcohol, and are also characterized by yielding much glutaminic acid, proline, and ammonia, and very little arginine and histidine, and not more than traces of lysine, other vegetable proteins which yield much arginine were tried for comparison. Edestin and squash-globulin were selected because they dissolve easily, and also because our previous anaphylactic experiments showed the former to be relatively inactive while the latter was extremely toxic to sensitized animals when injected intraperitoneally.

Experiment 11.—Two rabbits were immunized to edestin from hemp-seed, by intraperitoneal injections as follows: January 17, 0.3 gm.; January 21, 0.4 gm.; January 24, 0.4 gm.; January 27, 0.5 gm.; January 31, 0.5 gm.; February 3, 0.5 gm.; February 7, 0.5 gm. On February 28 the surviving animal was killed and the serum collected (the other had died after the fourth injection). The antiserum gave with edestin positive complement fixation and precipitin reactions, each in 1–100,000 dilution, as well as a very severe passive anaphylactic reaction. The precipitin reaction was also given with the closely related flax-seed globulin, in a 1–10,000 dilution, while the complement fixation was here negative. This is of interest in view of the observations of White and Avery, that 2 guinea-pigs sensitized with edestin reacted typically to flax-seed globulin, one fatally.

Experiment 12.—Six doses, containing a total of 2.8 gm. of squash-seed globulin, were injected intraperitoneally, at intervals of 3 or 4 days, into 2 rabbits, and 10 days after the last injection they were killed. Their serum then gave about an equal titre on a preliminary complement fixation test, which was positive, in 1–100,000 dilution; it also gave the precipitin test when squash-seed globulin was used as antigen. The passive anaphylactic reaction gave striking results, being severe with squash-seed globulin. When the chemically similar, but genetically distantly related excelsin from the Brazil-nut, *Bertholletia excelsa*, was used as antigen, the complement fixation reaction was positive only in 1–1,000, and the precipitin reaction in 1–10,000 dilution.

These results are in striking agreement with those obtained by anaphylaxis tests;[1] that is, of 11 guinea-pigs sensitized with squash-seed globulin, 7 reacted, 1 even fatally, when excelsin was subsequently injected. This forms a striking illustration of the fact emphasized in our previous articles, that specificity almost certainly depends upon chemical rather than on biological relationships of the proteins used.

The results of the last two experiments indicate that when the precipitin reaction is given with very dilute solutions, the serum will also readily produce passive anaphylaxis. The control experiment (No. 13) with egg-white also shows this. At the same time hordein immunization, which at first shows only the complement fixation test, when carried farther leads to the appearance of the precipitin and passive anaphylaxis reactions.

Experiment 13.—Control with egg-white. To control these experiments a rabbit was given, intraperitoneally, 57.5 c.c. of egg-white in 6 doses during 24 days, and 12 days after the last injection it was bled to death. A sample of serum drawn on the fifteenth day gave no precipitin reaction with egg-white in dilutions from 1–10 to 1–10,000. On the twenty-third day a positive precipitin reaction was given by dilutions of 1–10,000 and on the thirty-sixth day in dilutions of 1–200,000, while positive complement fixation was then obtained in dilutions of 1–10,000,000, the greatest dilution tried. Passive anaphylaxis was conferred upon guinea-pigs by intraperitoneal injection of doses of from 0.5 to 3.0 c.c. of the 36-day serum, all reacting severely and about alike when given 1 c.c. of egg-white intraperitoneally 48 hrs. later.

Experiment 14.—Control with human ascites fluid. A total of 52.5 c.c. of human ascites fluid was injected intravenously into a rabbit in 6 doses during 15 days, and 10 days after the last injection the animal was bled to death. This serum then gave a positive complement fixation reaction with ascites fluid in dilutions up to 1–1,000,000, and a precipitin reaction with dilutions up to 1–100,000. Passive anaphylaxis experiments were not very successful, for 8 guinea-pigs which had received injections of 0.5 to 3.0 c.c. of the serum showed only very slight symptoms when 1 c.c. ascites fluid was given 48 hrs. later.

The results of these experiments with vegetable proteins are summarized in Table 1, p. 372.

DISCUSSION OF RESULTS.

Summarizing these experiments, as detailed in the protocols and Table 1, we have the following observations:

Three rabbits immunized with wheat gliadin yielded a serum which gave a specific complement fixation reaction with wheat gliadin even in dilutions up to 1–100,000, but not with rye gliadin or hordein in dilutions of 1–2,000. These sera failed to

[1] *Jour. Infect. Dis.*, 1911, 8, p. 66.

TABLE 1.

Immunization		Complement Fixation			Precipitin Reaction		Passive Anaphylaxis	
Material	Route	Hordein	Gliadin Wheat	Gliadin Rye	Hordein	Gliadin Wheat	Hordein	Gliadin Wheat
Gliadin wheat—								
(1) 1.2 gm....	Vein	.....	.....	.....	.....	Negative	.....	.....
(2) 2.0 gm....	"	.....	Positive up to 1:4,000	.....	.....	Negative	.....	Negative
(3) 4.7 gm....	Peritoneum	Negative at 1:2,000	Positive up to 1:10,000	Negative at 1:2,000	.....	.....	.....	.....
(4) 4.7 gm....	"	Negative at 1:2,000; also negative to malt and wheat "proteoses"	Positive up to 1:100,000	Negative at 1:2,000	.....	Negative	.....	Negative
Hordein—								
(5) 2.6 gm....	Vein	Positive up to 1:400,000	Negative at 1:2,000	Negative at 1:2,000	Negative at 1:1,000	.....	Negative	.....
(6) 2.6 gm....	"	Positive up to 1:600,000	Negative at 1:2,000	Negative at 1:2,000	Negative at 1:1,000	.....	Negative	.....
(7) 3.5 gm....	"	Positive up to 1:200,000	Negative at 1:2,000	Negative at 1:2,000	.....	.....	.....	.....
(8) 3.5 gm....	"	Positive up to 1:300,000; negative to malt "proteose"	Negative at 1:2,000; also negative to wheat "proteose"	Negative at 1:2,000	Negative at 1:1,000	Negative at 1:2,000; also negative to rye gliadin	Negative	.....
(9) 5.85 gm...	Peritoneum	Positive up to 1:1,000,000; also positive to malt "proteose" 1:500,000	Positive up to 1:1,000,000; negative to wheat "proteose" 1:2,000	Positive up to 1:1,000,000	Positive at 1:100,000; negative to malt and wheat "proteose"	Positive at 1:200,000 and to rye gliadin 1:40,000	Slight reactions	Slight. With rye gliadin moderate reactions
(10) 5.8 gm...	"	Positive up to 1:500,000; also positive to malt "proteose" 1:500,000	Positive up to 1:1,000,000; also positive to wheat "proteose" 1:500,000	Positive up to 1:500,000	Positive at 1:40,000; negative to malt and wheat "proteose"	Positive at 1:4,000 to both wheat and rye gliadin	Slight reactions	Doubtful with both wheat and rye gliadin
Edestin—								
(11) 3.1 gm...	Peritoneum	Positive with edestin at 1:50,000 " " flax globulin at 1:10,000			Positive with edestin at 1:100,000 " " flax globulin at 1:10,000		Severe with edestin	
Squash globulin—								
(12) 2.8 gm...	"	Positive with squash globulin at 1:100,000 " " excelsin at 1:1,000			Positive with squash globulin at 1:100,000 Positive with excelsin at 1:10,000		Severe with squash globulin	

give positive precipitin reactions with wheat gliadin in 1–2,000 dilution, which is as strong a solution as usually can be used on account of the precipitate which often forms on mixing more concentrated solutions of gliadin with serum. The usual doses (0.5–3.5 c.c.) of these antisera did not render guinea-pigs passively anaphylactic to gliadin.

Since the preparations of hordein dissolved more easily than those of gliadin, we made most of our other experiments with this representative of the alcohol-soluble vegetable proteins. Three different sets of rabbits were immunized to hordein, one in the spring of 1912, one in the summer of 1912, and one in the winter of 1913. Each of these three sets received about the same number of injections during the same length of time, the first two intravenously, the last one intraperitoneally. The conditions under which these experiments were performed differed in these respects: (*a*) the time of year, (*b*) amount of material injected, and (*c*) possibly in the breed of rabbits. The first set, which received 2.63 gm. of hordein each, yielded an antiserum of high titre, as shown by the complement fixation reaction in dilutions of 1–400,000 to 1–600,000, specific in that it reacted only to hordein, and failed to react to the closely allied gliadin from wheat or rye. The precipitin and passive anaphylactic tests were negative with hordein.

The second set, receiving 3.5 gm. each, produced an antiserum which reacted similarly except that possibly there was a slight complement fixation reaction with wheat gliadin and malt proteose in 1:2,000 dilution.

Set 3, receiving 5.6 gm. each, showed an interesting change in the character of the resulting antiserum. In this case the antiserum for hordein gave the complement fixation reaction in relatively high dilution with the closely related heterologous proteins from wheat and rye and proteose from malt, but not with proteose from wheat. With the precipitin test, some degree of quantitative specificity was shown, the reaction being exhibited in highest dilution with hordein, in lower with wheat gliadin, in lowest with rye gliadin. Antiserum from one animal when injected into guinea-pigs sensitized them to hordein and to rye and wheat gliadin (passive anaphylaxis). Antiserum from another animal gave this passive

sensitization only for hordein. This latter serum also showed a similar definite gradation in the precipitin reaction; in both this and the complement fixation tests it had a lower titre than the first serum.

One rabbit was immunized to edestin from hemp-seed and another to the globulin from squash-seed. Since these proteins often cause death when injected intravenously,[1] we used intraperitoneal injections for the following experiments and thereby avoided all untoward symptoms. About 3 gm. of each of these two chemically similar proteins were given to each animal.

The antiserum obtained after immunizing with the globulin from the squash-seed gave positive reactions with the complement fixation, the precipitin, and the passive anaphylaxis tests. With the complement fixation test this serum reacted in dilutions not greater than 1:100,000, thus showing a much lower activity than the hordein antiserum which reacted in dilutions of 1:1,000,000; it, however, gave a severe passive anaphylactic reaction, whereas hordein antiserum gave only a mild one. It is interesting to note in this connection that the antiserum produced by the globulin of the squash-seed reacted with the excelsin from the Brazil-nut. This latter protein is similar to the globulin of the squash-seed, both in its physical properties and in the proportion of amino-acids yielded by hydrolyzing with strong acids. Here again we have another indication that these biological reactions are determined by the chemical constitution of the proteins.

Edestin immunization produced an antiserum which gave the complement fixation reaction in dilutions up to 1:50,000, the precipitin reaction at 1:100,000, and caused a strong passive sensitization.

The chief conclusions to be derived from these experiments are briefly as follows:

1. Carefully purified preparations of vegetable proteins readily produce antisera.

2. The antisera obtained in our experiments differed in their range of reactions, some giving only the complement fixation, some the complement fixation and precipitin tests, while others in addition conferred passive anaphylaxis to guinea-pigs.

[1] Autopsies show that the lung capillaries are occluded by granular matter (precipitated protein?).

3. Antisera to the same protein obtained from different individual animals differ in their reactions, for some unknown cause.

4. An antiserum at one stage of its development may be apparently of sharply limited specificity, giving only the complement fixation reaction with the homologous protein, while a sample taken later from the same animal, after the antibody content has increased, will react with heterologous proteins having similar chemical and physical properties.

5. At about the time the serum develops the complement fixation reaction with such heterologous proteins, the precipitin reaction as well as the passive anaphylaxis reactions appears, but at first may be limited to the homologous protein.

6. A specific complement fixation reaction in high dilution does not necessarily accompany reactions with the heterologous proteins, nor can such serum always produce the passive anaphylaxis reaction.

7. Both the precipitin and passive anaphylaxis reactions appear later in immunization than the complement fixation reaction, and seem to be closely related to each other in delicacy.

With these points in mind, it might be well to notice the relations of these reactions to those obtained by anaphylaxis alone, as described in our previous papers.

The first experiments, in which an antiserum was obtained that would react only with the homologous protein, seemed to show that the complement fixation reaction either is a more delicate reaction than anaphylaxis (which, with the same materials, gives reactions with related heterologous proteins), or that it is due to a different antibody. The later experiments show that when the animal is further immunized, positive complement fixation reactions can be obtained with closely related heterologous proteins, thus agreeing with the results of anaphylaxis experiments. At the time the precipitin reaction appears, the passive anaphylactic condition usually can be induced in guinea-pigs injected with this precipitating serum.

The important but insufficiently considered observations of Magnus[1] are fully confirmed by our present experiments with *pure proteins*. Magnus used extracts of plant tissues and found by

[1] *Ber deut. Bot. Gesellsch.*, 1908, 26*a*, p. 532.

carefully conducted precipitin tests that *the degree of immunization determines the range of reaction.* For example, when an animal is immunized but a short time with extracts of the seeds of one of the cereals, it yields a serum which precipitates only the extract of the same species; later in the course of immunization, precipitins appear for extracts of closely related species, and progressively a wider and wider list of cereals reacts, until finally precipitates are obtained with extracts of all the *Gramineae.* Nevertheless, even with this extreme degree of immunity the serum gave no reaction with extracts derived from plants not belonging to the *Gramineae.* As the order of appearance of reactions with heterologous extracts was always the same, Magnus holds that it is possible in this way to secure a standard for estimating the relationship of plants by biochemical means.

As to whether or not the antibody responsible for fixation of the complement is identical with that which causes the precipitin reaction, the anaphylactic phenomena, etc., we are unable at present to state, but inasmuch as the antisera which gave the precipitin test also caused passive anaphylaxis, it is possible that one and the same antibody is common to these two reactions. On the other hand, a larger amount of a common antibody may be required to produce these reactions than is required for the complement fixation reaction.

The questions raised by these experiments deserve further study and the accumulation of much more data before definite conclusions can be drawn. Unfortunately, our present knowledge of the actual chemical relations of different individual proteins to one another is too meager to serve as a guide in interpreting the results of such experiments, but it is not impossible that chemical relationships may be indicated by these biological reactions, which in conjunction with further chemical studies may ultimately lead to a better knowledge, not only of the nature of the processes causing these mysterious changes in the serum, but also of the chemical constitution of the proteins.